Final Research Report

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P.C. Thomas, J. van Dam, M. Kean and M. Gyimesi

CHRONIC TOXICITY OF [redacted] in DMP TO *DAPHNIA MAGNA* IN A 21 DAY REPRODUCTION TEST UNDER FLOW-THROUGH CONDITIONS

Project identification

Client	[redacted]	Client No	Project No	
Subclient	[redacted]	Subclient No	Task No	859.023

Drafted by department Environmental Chemistry and Regulatory Affairs

ABSTRACT

The purpose of this study was to assess the toxicity of [redacted] in DMP dissolved in fresh water, on the reproductive efficacy of *Daphnia magna* STRAUS - clone 5, in a 21-day flow-through test complying with the OECD Guideline No. 211, 21st September 1998 and EU guideline C.20 from Annex V of Directive 67/548/EEC.

The test criterion of toxicity used was reproductive capacity expressed as the number of neonates per daphnid per day.

The nominal concentrations used in the study were as follows:
0, 1.1, 2.25, 4.5, 9 and 18 mg/l

Analytical determinations of the test solutions were performed on 12 occasions during the test. The concentrations were found to remain stable to within 20% of the nominals over the test period. The nominal concentrations were used to calculate the effect concentrations.

The validity criteria were respected:
Mortality was <20% in the control group over the test period.
The average number of juveniles per replicate in the control was 1916 after 21 days equivalent to at least 95.8 neonates per daphnid. Due to the test design, the actual neonate production of individual parent animals could not be ascertained.

The No Observed Effect Concentration (NOEC) is determined as the concentration used in the study that is immediately below the Lowest Observed Effect Concentration (LOEC), the latter derived statistically from the data using the appropriate statistical test.

The test data for neonate production were found to be normally distributed and homogeneous. Using Dunnett's and Bonferroni-t tests, no significant differences were found. The No Observed Effect Concentration (NOEC) based on reproductive output, weight of adult daphnids and on parent body length was found to be ≥ 18.0 mg/l.

The EC_{50} for adult mortality and for reproduction could not be determined due to insufficient mortality in any of the test concentrations.
However, the EC_{10} value for parental mortality was found to be 9.7 mg/L. The final result for this study is therefore based on the EC_{10} for parental mortality of 9.7 mg/L

CHRONIC TOXICITY OF [redacted] in DMP TO *DAPHNIA MAGNA* IN A 21 DAY REPRODUCTION TEST UNDER FLOW-THROUGH CONDITIONS

Sponsors	[redacted]
Study monitor	C.L.J. Braun MD
STUDY ORGANISATION **Location**	[redacted]
Study director	Dr. P.C. Thomas
Quality Assurance Unit	Ing. H. van Daalen
Management	Ir. A.R. Luttmer
Experimental initiation date	26-11-2007
Experimental completion date	24-12-2007

ARCHIVING AND STORAGE

The project file including the final report, amendments to the final report, the study plan, amendments to the study plan, records of quality assurance inspections, all letters, memos and notes and raw data pertaining to the study will be retained in the archives of [redacted] for a period of ten years. Other records including master schedule sheet, laboratory notebooks, logbooks, records of the maintenance and calibration of equipment, summary of training, curricula vitae and job descriptions of the personnel involved in the study, records related to location and storage of the test substance will also be kept in the [redacted] archives for a period of ten years. Test material will be stored deep-frozen under the sample code [redacted] for ten years or only as long as the quality of the test substance permits evaluation.

GLP COMPLIANCE STATEMENT

The study was conducted in compliance with the following Good Laboratory Practice regulations:

- OECD Principles on Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98) 17.

The report contains an accurate description of the results.

Study director
Dr. P.C. Thomas

date 2.9.08

signature

Management
Ir. A.R. Luttmer

date 2/9/2008

signature 2/9/2008

voedsel en waren autoriteit

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC the conformity with the OECD Principles of GLP was assessed on 29-31 January 2008 at

Akzo Nobel Technology & Engineering BV
Environmental Chemistry & Regulatory Affairs Department
Velperweg 76
6824 BM Arnhem

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Physical-chemical testing, environmental toxicity studies on aquatic and terrestrial organisms and tests on behaviour in water, soil and air; bioaccumulation.

NEDERLAND

Den Haag, 19 March 2008

Dr Th. Helder
Manager GLP Compliance Monitoring Program

Food and Consumer Product Safety Authority (VWA)
Prinses Beatrixlaan 2, 2595 AL Den Haag
Postbus 19506, 2500 CM Den Haag
The Netherlands

QUALITY ASSURANCE STATEMENT

This report was audited by the Quality Assurance Unit contracted by Akzo Nobel Technology and Engineering Arnhem. It is considered to be an accurate presentation of the methods and procedures applied in the course of the study and an accurate reproduction of the data recorded.
Listed below are the dates of inspection of this study by the Quality Assurance Unit and the dates on which its findings were reported to Study Director and Management.

Dates of inspection	Phase of the study	Dates of reporting
25-11-2007	Study plan	25-11-2007
29-11-2007	Test substance stock solution	29-11-2007
03-12-2007	Selection and distribution of daphnids	03-12-2007
12-12-2007	Test: sampling and analysis	12-12-2007
01-08-2008	Final report	03-8-2008

Quality Assurance Unit
Ing. H. van Daalen

date 27. 8. 2008

signature

CONTENTS

1. INTRODUCTION

1.1 Objectives

The purpose of this study was to assess the toxicity of the test substance dissolved in fresh water, on the reproductive efficacy of *Daphnia magna* STRAUS - clone 5, in a 21-day flow-through test complying with the OECD Guideline No. 211, 21st September 1998 and EU guideline C.20 from Annex V of Directive 67/548/EEC.

The test criterion of toxicity used was reproductive capacity expressed as the number of neonates per daphnid per day and immobilisation of the neonates found in the test solutions. The No Observed Effect Concentration (NOEC) is determined as the concentration used in the study, which is immediately below the Lowest Observed Effect Concentration (LOEC), derived statistically from the data using analysis of variance. Further objectives such as the EC50 for adult mortality and for reproduction will be determined if applicable.

1.2 Principle of the test

Groups of daphnids divided into two replicates were exposed to the test substance added to test medium at a range of concentrations. Under otherwise identical test conditions the effects on adult mortality and reproduction of *Daphnia magna* exposed to the test substance were recorded over a period of 21 days.

2. TEST GUIDELINES, MODIFICATIONS AND DEVIATIONS

The study was carried out in accordance with OECD (8.1) Guidelines 211 for testing of chemicals (adopted September 1998) and EU guideline C.20 from Annex V of Directive 67/548/EEC without modification of the test guideline.

3. MATERIALS

3.1 Test substance

The test substance, project sample code T 07024 was supplied by the sponsor. Data on the handling, stability, composition, purity or other characteristics of the test substance supplied by the sponsor has been used without further verification. The analytical certificate is included in Appendix I.

• Product name	[redacted]n DMP (also known as [redacted] and [redacted])
• chemical name	The test substance is a preparation containing a mixture of the following methyl isopropyl ketone peroxides [redacted] in dimethylphtalate: Methyl isopropyl ketone peroxide Type 3 Methyl isopropyl ketone peroxide Type 4
• CAS reg. no.	Methyl isopropyl ketone peroxide Type 3 [redacted] Methyl isopropyl ketone peroxide Type 4 [redacted] Dimethylphthalate 131-11-3
• appearance	Transparent slightly viscous liquid
• water solubility	Greater than 100 mg/L [redacted]in DMP
• vapour pressure	Expected to be negligible
• batch number	BOE 07059
• stability	Stable when kept in a closed bottle in the refrigerator and away from light
• EC50 daphnids	34 mg/l based on measured concentrations
• storage until required	refrigerator / at room temperature away from light

3.2 Chemicals

All reagents used were of reagent grade quality and obtained from J.T. Baker Chemicals BV, Deventer, The Netherlands and Acros, Tilburg, The Netherlands or Fluka Chemie GmBH, CH-947 Buchs, Switzerland.

3.3 De-ionised water

The de-ionised water used in the study contained less than 10 µg/l of copper, with a conductivity of less than 5 µS/cm and less than 2.0 mg/l NPOC-content.

3.4 Test vessels

750 ml (nominal) glass aquaria were used, pierced at approximately 450 ml level with an overflow running to drain. The aquaria were filled by a flow-through system containing test solution which passed through glass tubing on the opposite side to the over-flow. The tubing used in the pumps was Viton tubing previously determined to be the best tubing for maintaining substance stability for this organic

peroxide. All remaining tubing was made of neoprene and glass in the test concentrations and silicone in the control. Joints were minimised where possible.

3.5 Test room, temperature control and light regime

The test was carried out in a temperature-controlled room. The test temperature was between 18 and 22°C and the actual temperature was kept constant within ± 1°C throughout the test. The light regime was 16 h of ambient light per day, provided by fluorescent tubes.

3.6 Apparatus

The dissolved oxygen concentrations were determined electrochemically using an oxygen electrode and meter. The pH was determined with a pH meter. The temperature was measured with a thermocouple and recorder and with a digital thermometer. The flow-through system consisted of multi head Gilson/Watson-Marlow peristaltic pumps set in parallel followed by *in situ* mixing of the stock solution to achieve appropriate dilutions.

3.7 Test medium

For the test solutions
Reconstituted water (M4) according to Elendt (1990) was used for culturing and in the test. The M4 was prepared as a 5 fold concentrated solution and then diluted by mixing the M4 super-stock with deionised water. The pH of the final solution was 8.0 ± 0.5 and the solution was made up following the guideline.

The five fold concentrated M4 sub-stock solution was prepared during weekdays from the original M4 stock components and the final M4 solution was made automatically *in situ* by pumping the sub-stock into deionised water in a 1 L mixing vessel. This vessel cascaded into a 30 L aquarium which was aerated, and this final M4 solution was used as the basis of the test solutions. Further to this algae were added to the M4 solutions in the 30 L aquarium using a pumping system.

For the stock solutions
M4 was used for the stock solutions as the stock solutions were left for periods up to 72 h and the test substance has previously been shown in a non-GLP test to be stable in M4 (<10% loss within 48 h).

3.8 Test animals

The test animals were taken from a *Daphnia magna* clone 5 stock, (origin: Notox b.v., Hambakenwetering 7, P.O. Box 3476, 5203 DL s'Hertogenbosch, The Netherlands). The animals used in the test were less than 24 hours old and were obtained from parent animals reproducing

parthenogenically and having an age of 2-4 weeks (having previously produced at least one brood before use).

4. METHODS

4.1 Test solutions

4.1.1 Preparation of the stock solutions

To prepare the stock solutions, 1.00 ± 0.003 g of the test substance was weighed out, then dissolved directly into 10 litres (determined using a measuring cylinder) of M4 (see § 3.7) in 10 litre Duran glass bottles and mechanically agitated using a magnetic stirrer. Previous non-GLP studies on stability have revealed that the test substance is stable for up to 72 h in M4. The obtained preparations was agitated mechanically for between 4 and 47 hours in an attempt to completely dissolve the test substance (previous non-GLP studies have shown that an aqueous solution of 100 mg/L of test substance in M4 can be obtained within one hour by mechanical agitation).
Test substance stocks were made as required, on the day they needed replacing (accept for 2 occasions where it was made earlier).The pH of each stock solution was checked and found to be between 8.1 and 8.5, therefore the pH was not adjusted.
A fresh stock solution was prepared for each solution change.

4.1.2 Preparation of the test solutions

Test solutions were prepared by further dilution of the stock solution with M4 under flow through conditions. The stock was pumped at a known rate into the dilution water and allowed to mix directly in the inlet pipe shortly before reaching the test aquaria. This minimised contact of the test substance with the algae thereby optimising stability.
A nominal geometric series of concentrations was used. The ratio between two consecutive concentrations was, nominally, 2.
Test vessels were filled directly by a flow-through system and analysed 3 times a week. Measured concentrations were used as feedback to immediately adjust the pumps of the flow-through system to maintain the concentrations within 80 and 120% of the nominal concentrations.
The pH of the test solutions was between 7.0 and 7.8 throughout the test, therefore no adjustments were made. One control containing only test medium was included in the test.
The test aquaria were replaced after 8 days and then whenever considered necessary by the technician, thereafter (based on visual observation of algal debris on the floor of the aquarium).

The test concentrations in the test were as follows:
0, 1.1, 2.25, 4.5, 9.0, 18.0 mg/L.

4.2 Test conditions

The test duration was fixed at 21 days. Two test vessels per concentration, with twenty daphnids per vessel, were tested at each test concentration and in dilution water to serve as a control. Test solutions were constantly renewed using a peristaltic pump system using a replacement rate of at least 10 volumes of the test vessels per day.

The test system was allowed to run prior to addition of the daphnids for 7 days until deemed to be stable (further to analytical measurements). The daphnids were randomly distributed to the test vessels and this was considered to be the beginning of the test.

4.3 Food

Culture animals were fed a diet, in the form of the algal strain *Chlorella vulgaris*. This strain is cultured in the ECRA Environmental Chemistry laboratory and the total organic carbon content to cell count ratio has previously been determined.

During the study the daphnids were continuously supplied with algae such that the cell count per mL was equivalent to the daily feeding rate recommended in the semi-static test of 0.1 mg of carbon per daphnid per day. This rate was maintained throughout the test for the control and the test solutions containing test substance.

The algae concentrations (cells/mL) in the controls and the test solutions were checked at least once per week. The concentration of cells provided to the daphnids ranged from 112 500 to 225 000 cells/ml while the target concentration was 200 000 cells/ml. This range was considered acceptable for the continued health of the daphnid population without leading to problems of over-feeding or significant loss of the test substance during the study.

4.4 Physico-chemical Parameters

Temperature, pH, dissolved oxygen concentration and conductivity were measured in the control solution and all test concentrations in which parent animals are living at day 0, 7, 14 and 21. Water hardness was measured at the end of the test in the control and the highest test concentration.
Temperature in a beaker placed near the test vessels was also monitored continuously.
Light intensity was measured once during the study.
pH: minimum 6 and maximum 9 and should not vary more than 1.5 units during the test.
Oxygen concentration: at least 3 mg/l throughout the test. Dilution water was aerated prior to the addition of the test substance but not during the test.
Temperature: between 18°C and 22°C but not varying by more than 2 °C throughout the test.

4.5 Inspection

Animals were checked for immobilisation of parent daphnids on at least six occasions per week of the test. From the day of the first brood, observations of broods (aborted, living and dead progeny) were also made in each container at each concentration. The day of brood release and the number of living and dead neonates per brood or abortions were noted. Any other abnormal observations were also recorded.

At the end of the test, the length of all surviving parent animals was measured to the nearest 0.1 unit using a binocular microscope. Following measurement of body length, parent animals were weighed (dry weight) by placing in an oven at 105°C overnight, individually, if possible, or per group (to calculate the mean individual weight per surviving parent animals per concentration).

4.6 Sampling

As the study substance is known to be unstable under the conditions of the study, samples were taken three times per week. Samples were filtered over a Pall 0.45 µm GHP Acrodisc filter, transferred into 10 ml HPLC vials, and analysed immediately. When considered necessary by the SD, further samples were taken within 24 hours, as described above, and analysed immediately.

4.7 Chemical analyses

The method used to determine the concentration of the test substance in the test medium is described in Annex 2.
Mean concentrations were calculated using a time weighted mean method found to be between 92.7% and 105.7% of the nominal concentration, therefore all results were based on nominal concentrations.

Validation
A calibration curve of the test substance was made in the concentration range 0 – 20 mg/L (n=7). From these results the limit of Detection and Quantification were calculated and found to be 0.064 mg/L and 0.21 mg/L, respectively. The calibration series was linear with a squared regression coefficient of 1. In Annex 2 a more detailed description can be found on the validation and calculation procedure.
Further preliminary assays, not attached to this study and also not performed following Good Laboratory Practice regulations, were carried out before the test using chemical analysis:

- to confirm the water solubility at the highest concentration to be prepared for the test (that of the stock solutions),
- to verify the stability of the test substance in the mineral media used in the study.

5. RESULTS

5.1 Preliminary test

Non-GLP preliminary studies were performed to determine the stability of the test substance and similar compounds in the medium, influence of algae concentration on the substance stability. Influence of a flow-through set up on daphnid growth and reproduction and toxicity of the test substance on the ECRA daphnid strain.
The results from these studies are included in a report (Thomas, *et al.*, 2007) and summarised below.

[redacted] was found to be stable for up to 72 hours at the stock concentration of 100 mg/L in the test medium. The effect of algae onto the stability could be significantly reduced by filtration, after filtration the stability of [redacted] is similar to that of the stock solution.

Results from internal studies were compared with those from the NOTOX report (Migchielsen, 2002)

5.2 Water quality and analytical results

Constant record of temperature over test time:
Temperature: between 18.4°C and 19.5°C throughout the test.
Oxygen concentration: min. 6.3 mg O_2/l; max. 8.9 mg O_2/l
Conductivity: between 504 and 700 µS/cm throughout the test.
Water hardness: 12.3 °dH in the control and 13.2 °dH in 18 mg/L on day 21.

Results of Physico-chemical parameter measurements are presented in Annex 3.

The test solutions were found to be stable over the test period. As concentrations were observed to be within ± 20% of the nominals, all statistical evaluation has been based on nominal concentrations. A full description of the analytical method and results table is provided in Annex 2.

5.3 Parent animal mortality

The following daphnids died in each of the following concentrations: 1, 2 and 3 in the control (on day 9, 16 and 18, respectively); 1 on five occasions in 1.1 mg/L (on day 8, 9, 11, 15 and 17, respectively); 1 on five occasions and then 2 in 2.25 mg/L (on day 9, 10, 11, 14, 16 and 17, respectively); 1 on three occasions and then 2 in 4.5 mg/L (on day 9, 14, 16 and 17, respectively); 1 in 9.0 mg/L (day 15); and 1, 2, 1, 5, 1, 1 and 1 in 18.0 mg/L (on day 3, 8, 10, 14, 15, 17 and 18, respectively). These mortalities were considered to be concentration related.

5.4 Coefficient of variation of control fecundity

The number of juveniles per replicate in each concentration is shown in table 2. The validity criteria for the coefficient of variation (less than 25% in the control based on the number of living neonates for each replicate at the end of the test) was achieved.

The full data record of neonates released per day is presented in Annex 4.

Table 2: Total number of juveniles per replicate at end of the test at each test concentration, total number of neonates per concentration and coefficient of variance.

	Concentration (mg/l)					
Rep no.	**0**	**1.1**	**2.25**	**4.5**	**9.0**	**18.0**
I	1978	1921	1935	2110	2323	1811
II	1854	1928	1838	1883	2100	1807
Total	3832	3849	3773	3993	4423	3618
CV (%)	4.6	0.3	3.6	8.0	7.1	0.2

5.5 Statistical evaluation of the reproduction data and length and weight of parent animals at end of study

The reproduction data was tested for normality using Chi-square test and found to be normally distributed. The data passed Bartlett's tests for homogeneity of variance. Analysis of variance was performed on the number of living neonates per replicate using the Bonferroni t-test and verified with a second multiple comparison method, the Dunnett's test (Annex 5).
In both of these tests no significant effect ($p>0.05$) compared to the control was observed at any of the concentrations.
All computations were performed using Toxstat version 3.0.

Further statistical analyses (using Toxstat version 3.0) were performed using length and weight data.

Both length and weight data were found to be normally distributed and displayed homogeneity of variance. Multi-comparison tests of group weights and length of parent animals were employed. No significant differences were found between the test concentrations and the control.
Based on results from reproductive output and parental length and weight, the No Observed Effect Concentration (NOEC) is equal to or greater than 18.0 mg/l.

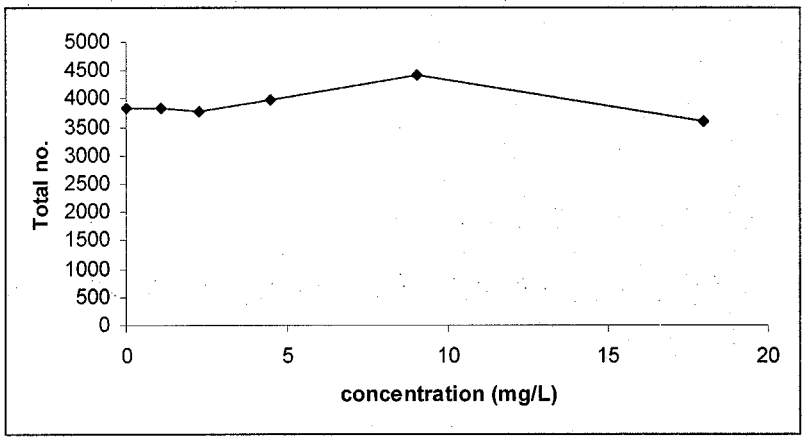

Figure 1: Plot of total number of neonates at each concentration

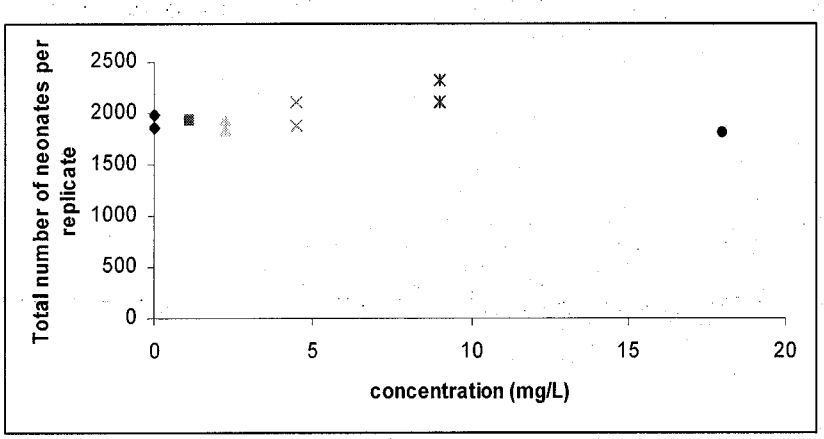

Figure 2: Plot of total number of neonates per replicate and per concentration

5.6 EC_{50} for parent animals

An EC_{50} based on reproduction capacity (or survival) of parent animals at the end of the test could not be calculated due to insufficient parent mortality.

As mentioned in section 5.3 parent mortality is considered to be concentration related; in the highest concentration tested 31% of the animals died.

Table 3: Survival of parent animals

Conc. (mg/L)	no. of daphnids at the start of the test		No. of surviving daphnids at the end of the test	
replicate	I	II	I	II
Control	20	20	17	17
1.1	20	20	17	18
2.25	18	20	17	14
4.5	20	20	20	15
9.0	20	20	20	19
18	21	18	14	13

An EC_{10} was determined by maximum likelihood regression using the probit transformation. Confidence limits could not be computed. All computations on survival were performed using the TOXCALC™ version 5.0 program.

Because of too high variation in the number of surviving animals between replicates and also between concentrations, the data were transformed. It was assumed that in the control no mortality occurred and all data were adjusted accordingly. This means that the number of animals at the start of the test were set to 17 for all concentrations and when the number of daphnids at the end of the test was higher than 17, this was also set to 17, which means no mortality. This transformation was used as a surrogate for the raw data making calculation of an EC_{10} possible. As the mortality data are clearly concentration related for the highest test concentration, this was considered the most appropriate statistical method to evaluate this endpoint.

The EC_{10} for survival is 9.7 mg/L.

5.7 Any other biological effects observed

No neonates were found immobile or dead during the test.

6. CONCLUSION

Data was found to be normally distributed and homogeneous. Using Dunnett's and Bonferroni-t tests, no statistical difference was found for reproductive output, parental body weight or length at any test concentration. The NOEC for all the reproductive endpoints is therefore ≥ 18.0 mg/l.

However, based on parental mortality during the test a concentration response relationship was found although 50% mortality was never reached in any concentration. The EC_{10} was calculated as 9.7 mg/L and therefore the final effect level is based on this value.

7. AMENDMENTS TO AND DEVIATIONS FROM THE STUDY PLAN

7.1 Amendments to the study plan

There were no amendments to the study plan.

7.2 Deviations from the study plan

- The obtained preparations were agitated mechanically for between 4 and 24 hours and on one occasion for 47 hours. In the study plan a stirring time between 2 and 4 hours is given. This deviation does not have an effect on the outcome of the test as can be seen from the measured concentrations which were not lower than 80% of the nominal concentration.
- Length of daphnids was not measured in mm but in units.
- Calibration curve until 20 mg/L, but some concentrations were above 20 mg/L.
- Erroneously the test concentrations mentioned in the study plan were without unit. It should have been in mg/L and this was used during the test and in the report.

8. QUALITY CRITERIA

The following quality criteria have been met:

- the mortality in the controls (parent females) should not exceed 20% at the end of the test. The mortality was 15%.
- the average cumulative number of living young produced per surviving parent female at the end of the test should be ≥ 60 in the controls. The average cumulative number of living young per parent (living and dead) was 95.8, if based on total neonates per group for all surviving parents in the control the cumulative number is 112.7

9. REFERENCES

8.1 OECD, (1998). Guidelines for testing of chemicals no. 211, Organisation for Economic Co-operation and Development, Paris.

8.2 Elendt B.P. (1990): Selenium deficiency in Crustacea, Protoplasma 154, 25 – 30

8.3 Toxstat version 3.0 (1989). Gulley DD, Boelter AM and Bergman HL. Dept of Zoology and Physiology, University of Wyoming.

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8.5 C.E. Stephan (1977) 'Methods for calculating an LC_{50}'. In: Aquatic toxicology and hazard evaluation, F.L. Mayer and J.L. Hamelink (Eds.), 65-84

8.6 Thomas P.C., Vos A., van Dam J., Kean M. & Helming B. (2007). Final interim report Development and validation of an analytical method for Butanox P50 and development and optimization of the flow through test set up for chronic daphnia and early life stage fish tests. Prelim study for T 07005 ODC & OFE.

8.7 Migchielsen M.H.J. (2002), Acute toxicity study in *Daphnia magna* with Trigonox R-938 (semi-static), Report no.: 338772.

8.8 Toxcalc™ v 5.0 (1994), Tidepool Scientific Software and Michael A Ives.

8.9 Toxstat version 3.0 (1989), Gulley D.D., Boelter A.M. and Bergman H.L., Dept of Zoology and Physiology, University of Wyoming.

ANNEX 1

[redacted]

Certificate of Analysis

[redacted]

Product name : -

Chemical name : [redacted]

Batch number : [redacted]

Test results:

Method	Analysis of	Unit	Result *1
Jo/72.10, Jo/72.11, Jo/02.1	Peroxidic compounds (sum) *See page 2 for a specification*	% m/m	28.8 (± 1.0)
HPLC	Dimethylphthalate IUPAC: Dimethyl 1,2-benzenedicarboxylate	% m/m	68.0 (± 1.0)
HPLC	[redacted]	% m/m	1.4 (± 0.2)
Amp/88.9	Water	% m/m	1.8 (± 0.2)

*1 bracketed values are estimated 95% confidence intervals

Archive code : TNA-2007008

Analytical documentation : MIRA-20070927

[redacted]

[redacted]

Certificate of Analysis

[redacted]

[redacted] batch [redacted]

specification of the peroxidic compounds

structure	% m/m
[redacted]	

[redacted]

ANNEX 2

Description of the analytical procedure for the quantification of Trigonox using a HPLC system

1. Introduction

A method is described to determine the concentration of Trigonox in water. Procedures and instrumentation are based on High Performance Liquid Chromatography combined with on-line Solid Phase Extraction and UV detection. Analysis is based on two peaks, i.e. [redacted] Type 3 and [redacted] Type 4, representing the active ingredient of the test substance. The concentration of the test substance in the analytical method is calculated as the sum of these 2 peaks. Samples were quantified using a calibration curve.

2. Analytical procedure

The following conditions were found to be suitable for the determination of the test compound for concentrations of 0.5 to 100 mg/l in de-ionised water, Dutch Standard Water and M4 medium.

- Autosampler: Spark, model Triathlon
- Pump: Knauer Smartline 1000
- Gradient manager: Knauer Smartline 5000
- Mobile phase:

0 min.	30% A	70% B
5 min.	30% A	70% B
15 min.	100% A	0% B
17 min.	100% A	0% B
18 min.	30% A	70% B
20 min.	30% A	70% B

  A= Acetonitrile B= HPLC water
- Column: Waters Symmetry 4,6 x 150mm 5µm C18 RP column, with guard column.
- On-line SPE cartridge: PLRP-s 15-25µm
- Flow rate: 1.0 ml/min
- Detector: UV/VIS detector, Applied Biosystems, model 759 A
- Wavelength: 220 nm
- Injection volume: 8 ml (trapped on SPE cartridge in 4 min. with flow of 2 ml/min.)
- Integrator: VG Chromatography server
- Integration software: Atlas 2002R1 v. 6.18

For preparing the standards at the beginning of the test period a stock solution of test substance in de-ionized water was made. For the calibration series dilutions in de-ionized water in a concentration range of 0 – 100 mg/L ($n \geq 5$) from the stock solution were made. The samples taken during the test were quantified using this calibration series.

During the test period every week a fresh stock solution of test substance was prepared. Before every analysis series a control sample from the middle range of the calibration standards, prepared from the stock solution of test substance of the current week, was analyzed. This control standard was analyzed at the beginning of every sample series and at a minimum rate of one per ten samples and at least at the end of each sample series.

3 Calculation of concentrations

Quantification was done by measurement of peak areas. The concentrations of the test substance in the samples were calculated from the relation between concentration of standards (Cs) and peak area (PAs) obtained with linear regression analysis:

PAs = Cs * Slope + constant

$$\text{Sample concentration} = \frac{\text{Sample peak area} - \text{constant}}{\text{slope}}$$

As peak area of the test substance the sum of the peak areas from the two components, [redacted] Type 3 and [redacted] Type 4, was considered.

4. Reproducibility and validation

With the system described above, the two components, considered to represent the test substance, eluted after about 15 minutes.

The analytical method was found to be linear over the concentration range of 0.5 to 100 mg/l of the test substance, using the conditions described above. Every separate HPLC calibration series should give a linear regression with a squared regression coefficient $r^2 \geq 95\%$ (n>=5). Control standards analyzed during the analyses should be within 10% of the expected values based on the calibration curve. If this was not the case a second control standard was analysed. If this standard still showed a deviation of ≥10% of the expected value, the calibration procedure was repeated.

Table 1: Calibration standards of the test substance

calibration sample	Concentration (mg/L)	Peak Area (µVs)
ST 0	0	0
ST 0.5	0.5	11892
ST 1.0	1	23212
ST 2.0	2	46772
ST 5.0	5	122407
ST 10	10	247537
ST 20	20	495554

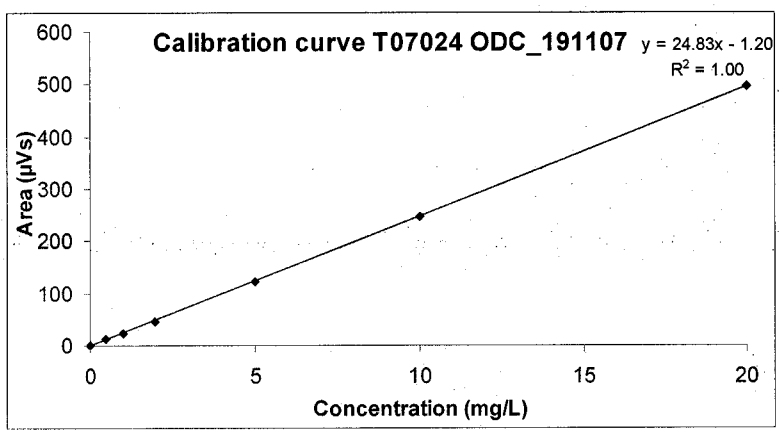

Figure 1: calibration curve of the test substance in deionised water

5. Results of measured test concentrations

Table 2: concentration of the time weighted average

Sample	Concentration (mg/L)	% of nominal
1.1 mg/L I	1.08	98.6
1.1 mg/L II	1.12	102.1
2.25 mg/L I	2.30	102.2
2.25 mg/L II	2.09	92.8
4.5 mg/L I	4.37	97.0
4.5 mg/L II	4.19	93.1
9 mg/L I	8.62	95.7
9 mg/L II	8.73	97.0
18 mg/L I	19.03	105.7
18 mg/L II	17.79	98.8

Table 3: measured concentrations per replicate during the test in mg/L

Day	1.1 mg/L I	1.1 mg/L II	2.25 mg/L I	2.25 mg/L II	4.5 mg/L I	4.5 mg/L II	9.0 mg/L I	9.0 mg/L II	18 mg/L I	18 mg/L II
1	1.20	1.27	3.77	2.38	4.88	5.95	9.33	8.90	22.47*	23.60*
2	0.93	0.92	2.60	1.57	3.44	3.59	8.56	8.22	16.76	16.30
3			2.68	1.72	4.39	4.65				
4	0.90	0.87	2.69	3.00	4.65	3.80	7.92	8.89	17.93	17.31
7	1.02	1.25	2.06	1.93	3.52	3.66	7.96	8.71	15.77	16.71
8	1.37				5.50					
9	1.15	1.29	2.24	2.15	4.62	4.72	9.26	9.79	21.55*	16.74
11	1.30	1.32	2.59	2.13	4.85	5.10	9.12	10.41	20.21*	16.69
14	0.87	1.00	1.90	1.75	2.67	2.82	8.17	8.33	19.02	14.19
15	1.28			2.30	4.49	5.29				22.68*
18	1.06	1.12	2.18	1.80	4.65	3.60	8.72	8.01	20.41*	19.67
21	1.06	1.22	1.99	2.18	5.22	4.35	9.77	7.40	18.86	17.67

* Highest concentration in the calibration curve was 20 mg/L, some of the analyzed samples during the test were above this value. However a preliminary (non GLP) test showed a linear calibration curve in the range of 0 to 100 mg/L. Therefore, the concentration is thought to be accurate, the high concentration can be explained by the fact that the tubes in the peristaltic pumps, delivering the stock and dilution media, varied in the actual volume they delivered over time (i.e. pumps were occasionally adjusted during the experiment).

Limit of Detection and Quantification

$$\text{Limit of detection} = \frac{3 * \text{standard error of calibration curve}}{\text{slope from the calibration curve}}$$

$$\text{Limit of quantification} = \frac{10 * \text{standard error of calibration curve}}{\text{slope from the calibration curve}}$$

ANNEX 3

Results of physico-chemical parameters measurements

Table 1: pH values

Day of test Concentration (mg/L)	0 hours	Day 7	Day 14	Day 21
Control	7.7	7.5	7.0	7.2
1.1	7.7	7.5	7.1	7.1
2.25	7.7	7.5	7.1	7.0
4.5	7.8	7.4	7.0	7.0
9.0	7.8	7.4	7.1	7.0
18.0	7.8	7.4	7.0	7.0

Table 2: Dissolved oxygen concentration in mg/L

Day of test Concentration (mg/L)	0 hours	Day 7	Day 14	Day 21
Control	8.6	8.9	8.3	8.3
1.1	8.6	8.5	7.7	7.6
2.25	8.4	8.6	8.0	7.4
4.5	8.5	7.9	7.5	6.5
9.0	8.5	7.8	7.2	6.3
18.0	8.4	7.6	7.6	6.7

Table 3: Conductivity in µs/cm

Day of test Concentration (mg/L)	0 hours	Day 7	Day 14	Day 21
Control	625	700	610	558
1.1	641	699	613	562
2.25	646	700	612	504
4.5	649	699	624	572
9.0	659	698	619	583
18.0	667	692	630	609
Standard m4 in *daphnia* culture	-	649	-	600

Table 4: Hardness in °dH

Day of test Concentration (mg/L)	Day 21
Control	12.3
18	13.2

ANNEX 4

Full data record of neonates released per day

Table 1: CONTROL

Day of Study	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Replicate No.	**Number of juveniles**																						**Total**
1 (20 adults at start)	0	0	0	0	0	0	0	76	-	377	-	279	-	-	353	-	232	-	261	-	-	400	1978
2(20 adults at start)	0	0	0	0	0	0	0	104	-	328	-	257	-	-	255	-	219	-	386	-	-	305	1854
Mortality of parental 1 [N]	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	3
Mortality of parental 2 [N]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	3
Total mortality of parent groups [%]	0	0	0	0	0	0	0	0	0	2.5							7.5		15				15%
Immobile / Stillborn juveniles	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

– = no juveniles counted

Table 2: 1.1 mg/l

Day of Study	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Replicate No.	**Number of juveniles**																						**Total**
1 (20 adults at start)	0	0	0	0	0	0	0	33	-	366	-	307	-	-	209	-	172	-	439	-	-	395	1921
2(20 adults at start)	0	0	0	0	0	0	0	41	-	336	-	281	-	-	289	-	141	-	428	-	-	412	1928
Mortality of parent group1 [N]	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	3
Mortality of parental 2 [N]	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	2
Total mortality of parent groups [%]	0	0	0	0	0	0	0	0	2.5	5.0		7.5				10		12.5					12.5%
Immobile / Stillborn juveniles	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

– = no juveniles counted

Table 3: 2.25 mg/l

Day of Study	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Replicate No.	**Number of juveniles**																						**Total**
1 (18 adults at start)	0	0	0	0	0	0	0	23	-	372	-	264	-	-	217	-	209	-	386	-	-	464	1935
2(20 adults at start)	0	0	0	0	0	0	0	31	-	325	-	244	-	-	252	-	228	-	356	-	-	402	1838
Mortality of parent group 1[N]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Mortality of parent group 2 [N]	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0	2	0	0	0	0	6
Total mortality of parent groups [%]	0	0	0	0	0	0	0	0	0	2.6	5.2	7.8			10.5		13.1	18.4					18.4%
Immobile / Stillborn juveniles	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

– = no juveniles counted

Table 4: 4.5 mg/l

Day of Study	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Replicate No.	**Number of juveniles**																						**Total**
1 (20 adults at start)	0	0	0	0	0	0	0	41	-	431	-	297	-	-	246	-	151	-	417	-	-	527	2110
2(20 adults at start)	0	0	0	0	0	0	0	30	-	394	-	390	-	-	152	-	131	-	337	-	-	449	1883
Mortality of parental group 1 [N]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mortality of parental group 2 [N]	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	2	0	0	0	0	5
Total mortality of parent groups [%]	0	0	0	0	0	0	0	0	0	2.5					5.0		7.5	12.5					12.5%
Immobile / Stillborn juveniles	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

– = no juveniles counted

Table 5: 9.0 mg/l

Day of Study	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Replicate No.	**Number of juveniles**																						**Total**
1 (20 adults at start)	0	0	0	0	0	0	0	76	-	339	-	441	-	-	192	-	149	-	429	-	-	697	2323
2(20 adults at start)	0	0	0	0	0	0	0	69	-	422	-	423	-	-	220	-	133	-	318	-	-	515	2100
Mortality of parent group 1[N]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mortality of parent group 2 [N]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Total mortality of parent groups [%]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5							2.5%
Immobile / Stillborn juveniles	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

– = no juveniles counted

Table 6: 18.0 mg/l

Day of Study	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Replicate No.	**Number of juveniles**																						**Total**
1 (21 adults at start)	0	0	0	0	0	0	0	102	-	233	-	399	-	-	121	-	169	-	252	-	-	535	1811
2(18 adults at start)	0	0	0	0	0	0	0	110	-	216	-	412	-	-	87	-	207	-	284	-	-	491	1807
Mortality of parent group 1 [N]	0	0	0	0	0	0	0	0	2	0	1	0	0	0	2	1	0	0	1	0	0	0	7
Mortality of parent group 2 [N]	0	0	0	1	0	0	0	0	0	0	0	0	0	0	3	0	0	1	0	0	0	0	5
Total mortality of parent groups [%]	0	0	0	2.6					7.7		10.2				23.0	25.6		28.2	30.7				30.7%
Immobile / Stillborn juveniles	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

– = no juveniles counted

Table 7: Length of adult Daphnids in unconverted binocular scale units

Daphnia No.	0 mg/L		1.1 mg/L		2.25 mg/L		4.5 mg/L		9.0 mg/L		18.0 mg/L	
	I	II	I	II	I	II	I	II	I	II	I	II
1	2.7	3.1	3.1	3.2	2.6	3.0	3.0	3.0	2.9	3.1	3.2	3.0
2	3.0	3.1	2.9	3.1	3.2	2.9	2.9	2.8	2.8	3.1	3.0	3.1
3	2.9	2.9	3.2	2.9	3.1	3.1	2.9	3.0	2.9	3.0	2.9	3.1
4	2.9	3.1	2.9	3.0	3.0	3.1	2.9	3.0	3.1	2.9	2.9	3.0
5	3.1	3.0	3.0	2.8	3.2	3.1	2.9	2.9	2.7	2.9	2.8	2.9
6	3.2	3.1	2.9	3.0	2.8	3.1	2.9	3.0	3.2	2.7	2.9	3.0
7	3.0	3.0	3.2	2.8	3.0	3.0	2.9	2.8	2.8	3.0	2.7	2.9
8	3.2	2.9	3.2	2.8	3.0	3.2	2.9	2.9	3.1	3.0	2.6	3.0
9	3.0	3.1	2.9	3.2	2.9	3.0	3.0	3.0	3.0	2.7	3.0	3.3
10	3.1	2.9	3.1	2.9	3.3	2.8	3.1	3.0	3.1	3.1	2.7	2.9
11	3.0	3.1	3.0	3.1	3.1	2.8	3.1	2.8	2.9	3.0	2.7	2.9
12	3.0	3.1	2.8	3.0	3.0	3.1	3.2	3.2	2.9	2.7	3.0	3.0
13	2.9	3.2	2.8	3.2	3.0	3.2	3.0	2.7	3.0	2.8	3.1	3.1
14	3.1	3.1	3.1	3.1	3.2	2.7	3.1	2.9	3.1	2.7	3.1	-
15	3.0	3.0	2.9	2.8	3.2	-	3.1	2.8	2.7	2.9	-	-
16	3.0	3.1	2.8	3.0	3.2	-	2.6	-	3.1	3.0	-	-
17	2.8	3.0	3.2	3.1	3.2	-	2.9	-	3.1	3.1	-	-
18	-	-	-	3.1	-	-	3.1	-	3.3	2.9	-	-
19	-	-	-	-	-	-	3.1	-	2.9	2.7	-	-
20	-	-	-	-	-	-	2.9	-	3.0	-	-	-
mean	3.0	3.0	3.0	3.0	3.1	3.0	3.0	2.9	3.0	2.9	2.9	3.0

Table 8: Parental weight (dry)

Concentration (mg/L)	replicate	No. of Daphnids	Total weight (g)	Mean weight per daphnid (mg)
control	I	17	0.0073	0.429
	II	17	0.0081	0.476
1.1	I	17	0.0068	0.400
	II	18	0.0077	0.428
2.25	I	17	0.0077	0.453
	II	14	0.0055	0.393
4.5	I	20	0.0115	0.575
	II	15	0.0099	0.660
9.0	I	20	0.0109	0.545
	II	19	0.0118	0.621
18.0	I	14	0.0092	0.657
	II	13	0.0066	0.508

ANNEX 5

STATISTICAL RESULTS

Results on number of neonates per replicate

Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	0.804	2.904	4.584	2.904	0.804
OBSERVED	0	6	0	6	0

Calculated Chi-Square goodness of fit test statistic = 12.7934
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance

Calculated B statistic = 9.07
Table Chi-square value = 15.09 (alpha = 0.01)
Table Chi-square value = 11.07 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 1.00
Used for Chi-square table value ==> df (#groups-1) = 5

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is used to calculate the B statistic (see above).

Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	191882.667	38376.533	3.652
Within (Error)	6	63054.000	10509.000	

Total 11 254936.667

Critical F value = 4.39 (0.05,5,6)
Since F < Critical F FAIL TO REJECT Ho:All groups equal
Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	control	1916.000	1916.000		
2	1.1 mg/l	1924.500	1924.500	-0.083	
3	2.25 mg/l	1886.500	1886.500	0.288	
4	4.5 mg/l	1996.500	1996.500	-0.785	
5	9.0 mg/l	2211.500	2211.500	-2.883	
6	18.0 mg/l	1809.000	1809.000	1.044	

Dunnett table value = 2.83 (1 Tailed Value, P=0.05, df=6,5)

Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	control	2			
2	1.1 mg/l	2	290.113	15.1	-8.500
3	2.25 mg/l	2	290.113	15.1	29.500
4	4.5 mg/l	2	290.113	15.1	-80.500
5	9.0 mg/l	2	290.113	15.1	-295.500
6	18.0 mg/l	2	290.113	15.1	107.000

Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	191882.667	38376.533	3.652
Within (Error)	6	63054.000	10509.000	
Total	11	254936.667		

Critical F value = 4.39 (0.05,5,6)
Since F < Critical F FAIL TO REJECT Ho:All groups equal

Transform: NO TRANSFORMATION

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	control	1916.000	1916.000		
2	1.1 mg/l	1924.500	1924.500	-0.083	
3	2.25 mg/l	1886.500	1886.500	0.288	
4	4.5 mg/l	1996.500	1996.500	-0.785	
5	9.0 mg/l	2211.500	2211.500	-2.883	
6	18.0 mg/l	1809.000	1809.000	1.044	

Bonferroni T table value = 3.14 (1 Tailed Value, P=0.05, df=6,5)

Transform: NO TRANSFORMATION

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	control	2			
2	1.1 mg/l	2	322.200	16.8	-8.500
3	2.25 mg/l	2	322.200	16.8	29.500
4	4.5 mg/l	2	322.200	16.8	-80.500
5	9.0 mg/l	2	322.200	16.8	-295.500
6	18.0 mg/l	2	322.200	16.8	107.000

Results on parental length

Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	13.534	48.884	77.164	48.884	13.534
OBSERVED	17	33	86	56	10

Calculated Chi-Square goodness of fit test statistic = 9.0193
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance

Calculated B statistic = 6.59
Table Chi-square value = 15.09 (alpha = 0.01)
Table Chi-square value = 11.07 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 32.67
Used for Chi-square table value ==> df (#groups-1) = 5

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is used to calculate the B statistic (see above).

Transform: NO TRANSFORM

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	0.252	0.050	2.381
Within (Error)	196	4.170	0.021	
Total	201	4.422		

Critical F value = 2.29 (0.05,5,120)
Since F > Critical F REJECT Ho:All groups equal

Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	control	3.021	3.021		
2	1.1 mg/l	3.003	3.003	0.508	
3	2.25 mg/l	3.035	3.035	-0.414	
4	4.5 mg/l	2.951	2.951	1.982	
5	9.0 mg/l	2.947	2.947	2.162	
6	18 mg/l	2.956	2.956	1.741	

Bonferroni T table value = 2.36 (1 Tailed Value, P=0.05, df=120,5)

Transform: NO TRANSFORM

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	control	34			
2	1.1 mg/l	35	0.082	2.7	0.018
3	2.25 mg/l	31	0.085	2.8	-0.015
4	4.5 mg/l	35	0.082	2.7	0.069
5	9.0 mg/l	40	0.080	2.6	0.073
6	18 mg/l	27	0.088	2.9	0.065

Results on parental weight

Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	0.804	2.904	4.584	2.904	0.804
OBSERVED	0	6	0	6	0

Calculated Chi-Square goodness of fit test statistic = 12.7934
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance

Calculated B statistic = 2.15
Table Chi-square value = 15.09 (alpha = 0.01)
Table Chi-square value = 11.07 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 1.00
Used for Chi-square table value ==> df (#groups-1) = 5

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is used to calculate the B statistic (see above).

Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	0.084	0.017	5.667
Within (Error)	6	0.021	0.003	
Total	11	0.105		

Critical F value = 4.39 (0.05,5,6)
Since F > Critical F REJECT Ho:All groups equal

Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	control	0.453	0.453		
2	1.1 mg/l	0.414	0.414	0.703	
3	2.25 mg/l	0.423	0.423	0.539	
4	4.5 mg/l	0.618	0.618	-3.012	
5	9.0 mg/l	0.583	0.583	-2.383	
6	18 mg/l	0.583	0.583	-2.373	

Dunnett table value = 2.83 (1 Tailed Value, P=0.05, df=6,5)

Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	control	2			
2	1.1 mg/l	2	0.155	34.3	0.039
3	2.25 mg/l	2	0.155	34.3	0.030
4	4.5 mg/l	2	0.155	34.3	-0.165
5	9.0 mg/l	2	0.155	34.3	-0.131
6	18 mg/l	2	0.155	34.3	-0.130

Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	0.084	0.017	5.667
Within (Error)	6	0.021	0.003	
Total	11	0.105		

Critical F value = 4.39 (0.05,5,6)
Since F > Critical F REJECT Ho:All groups equal

Transform: NO TRANSFORMATION

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	control	0.453	0.453		
2	1.1 mg/l	0.414	0.414	0.703	
3	2.25 mg/l	0.423	0.423	0.539	
4	4.5 mg/l	0.618	0.618	-3.012	
5	9.0 mg/l	0.583	0.583	-2.383	
6	18 mg/l	0.583	0.583	-2.373	

Bonferroni T table value = 3.14 (1 Tailed Value, P=0.05, df=6,5)

Transform: NO TRANSFORMATION

BONFERRONI T-TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	control	2			
2	1.1 mg/l	2	0.172	38.0	0.039
3	2.25 mg/l	2	0.172	38.0	0.030
4	4.5 mg/l	2	0.172	38.0	-0.165
5	9.0 mg/l	2	0.172	38.0	-0.131
6	18 mg/l	2	0.172	38.0	-0.130